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Growth factors delivery from hybrid PCL-starch scaffolds processed using supercritical fluid technology☆

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ABSTRACT

Synthetic polymeric scaffolds to be used as surrogates of autologous bone grafts should not only have suitable physicochemical and mechanical properties, but also contain bioactive agents such as growth factors (GFs) to facilitate the tissue growth. For this purpose, cost-effective and autologous GFs sources are preferred to avoid some post-surgery complications after implantation, like immunogenicity or disease transmission, and the scaffolds should be processed using methods able to preserve GFs activity. In this work, poly(ε-caprolactone) (PCL) scaffolds incorporating GFs were processed using a green foaming process based on supercritical fluid technology. Preparation rich in growth factors (PRGF), a natural and highly available cocktail of GFs obtained from platelet rich plasma (PRP), was used as GF source. PCL:starch:PRGF (85:10:5 weight ratio) porous solid scaffolds were obtained by a supercritical CO_2-assisted foaming process at 100 bar and 37 °C with no need of post-processing steps. Bioactivity of GFs after processing and scaffold cytocompatibility were confirmed using mesenchymal stem cells. The performance of starch as GF control release component was shown to be dependent on starch pre-gelification conditions.

1. Introduction

Under healthy conditions, bone has the ability of self-repair unless the separation between the broken pieces exceeds a certain threshold. In the latter case, natural or synthetic grafts are required to act as bridges and facilitate bone regeneration. Autologous bone grafting is a common choice for long defect tissue repairs to ensure biocompatibility and osteogenicity and to avoid immunogenicity. However, this practice is associated to pain and morbidity from the second surgical (donor) site (in *ca.* 20% of the cases) with the subsequent slow recovery, prolonged hospital periods and high sociosanitary costs (Malhotra, Pelletier, Oliver, Christou, & Walsh, 2014; Myeroff & Archdeacon, 2011). To overcome these limitations, novel alternative approaches to autografts in the form of synthetic scaffolds are thus being prospected for the clinical treatment of severe bone trauma.

Synthetic scaffolds should be designed to provide a suitable environment for recruitment of host cells and guide the growth of new tissue. Osteoconductive ability can be conferred to synthetic scaffolds by the addition of inorganic admixtures (e.g., hydroxyapatite, tricalcium phosphate) and/or organic signalling compounds (growth factors, GFs) (Bose & Tarafder, 2012; Lieberman, Daluiski, & Einhorn, 2002; Shrivats, McDermott, & Hollinger, 2014; Wang et al., 2014). Namely, the incorporation of growth factors in the graft regulates tissue formation by enhancing cell ingrowth, differentiation into the intended cell type and tissue growth. The elevated costs and safety concerns associated to human recombinant GFs have prompted the investigation on PRP and derivative preparations as a natural source of growth and differentiation factors suitable for soft tissue healing inducing collagen production (Alsousou, Thompson, Hulley, Noble, & Willett, 2009; De Pascale, Sommese, Casamassimi, & Napoli, 2015; Horimizu et al., 2013; Klein, Yalamanchi, Pham,

Abbreviations: BCA, bicinchoninic acid assay; BMP, bone morphogenetic protein; BSA, bovine serum albumin; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GF, growth factor; MSCs, mesenchymal stem cells; PBS, phosphate buffer solution; PCL, poly(ε-caprolactone); PDGF-BB, platelet-derived growth factor (isoform BB); PLGA, poly(lactic-co-glycolic acid); PLLA, poly(L-lactic acid); PRP, platelet-rich plasma; PRGF, preparation rich in growth factors; $scCO_2$, supercritical carbon dioxide; sc-drying, supercritical drying; St, starch; SEM, scanning electron microscopy; T_m, melting temperature.

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Longaker, & Chan, 2002; Roukis, Zgonis, & Tiernan, 2006). Moreover, the synergistic effect of these GFs is also stated to stimulate cell proliferation and to induce bone tissue formation, although contrasting results of *in vitro*, animal and clinical studies are reported in the literature likely due to the lack of standard PRP/PRGF production methods and improper uses (Alsousou et al., 2009; De Pascale et al., 2015; Graziani et al., 2006; Kasten et al., 2008; Lowery, Kulkarni, & Pennisi, 1999; Malhotra et al., 2014; Roukis et al., 2006). Finally, concerns on immunogenic reactions and transmissible diseases would be avoided if autologous PRP/PRGF were used in the graft. Among the different cytokines included in PRP/PRGF, some of them like platelet derived growth factor (PDGF), transforming growth factor (TGF-β1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (Reed, 2004) play a main role in cell recruitment, proliferation and differentiation.

GF-containing scaffolds able to efficiently provide a sustained release maintaining the physiological GF levels in a long-term are highly ambitioned. Soaking of the scaffold in a bone morphogenetic protein (BMP) solution just before implantation (Blackwood, Bock, Dargaville, & Woodruff, 2012), despite being clinically practiced, is regarded as inefficient (poor GF release control leading to concentrations much higher than physiological levels) and expensive (high content of GFs of recombinant origin). The occurrence of complications related to ectopic bone formation has also been reported (Blackwood et al., 2012; Carragee, Hurwitz, & Weiner, 2011). The binding of the bioactive factors to the scaffold is important to reach the release profiles needed to mimic the natural sequences of tissue morphogenesis or regeneration, avoiding potential systemic toxicity problems.

Several processing techniques (electrospinning, freeze-drying, particle leaching, electrospraying, sol–gel) are being tested for the production of GF-releasing scaffolds with promising results (Clark, Milbrandt, Hilt, & Puleo, 2014; Diaz-Gomez et al., 2014; Díaz-Gómez, Ballarin, Abraham, Concheiro, & Alvarez-Lorenzo, 2015; Goh, Shakir, & Hussain, 2013; Kim et al., 2014; Li, Kaplan, & Zreiqat, 2014). Nevertheless, constraints in the use of these techniques stem from different processability limitations depending on the process ranging from restriction to hydrophilic polymer matrices and scaffold cytotoxicity caused by remnants of organic solvents to loss of GF activity due to denaturation under high temperature and mechanical stress or low GF entrapment yield due to washing and leaching steps. Processing with supercritical fluids, mainly $scCO_2$, arises as an alternative green technological platform for the preparation of scaffolds in a simple (few processing steps), economical (no downstream processes needed) and reproducible (software-assisted monitoring of critical variables) manner (García-González, Concheiro, & Alvarez-Lorenzo, 2015; García-González, Díaz-Gómez, Concheiro, & Alvarez-Lorenzo, 2015). The portfolio of supercritical processing techniques includes the supercritical foaming using $scCO_2$, which allows the preparation of scaffolds under mild operating conditions, avoids the use of organic solvents and retains the bioactivity of the GFs close to 100% (De Ponti, Lardini, Martini, & Torricelli, 1991; Howdle et al., 2001; Kanczler et al., 2008).

The ease of processing and excellent biocompatible and mechanical properties of polyesters (e.g., PCL, PLGA, PLLA) for scaffolds contrast with their inertness and hydrophobic behaviour, thus hindering wettability, cellular adhesion and interaction with hydrophilic active agents such as GFs. Combinations of polyesters with other polymers would lead to a hybrid material with tuneable physical and mechanical properties, degradation rates and bioactive agent release profiles (Sahoo, Toh, & Goh, 2010; Steele et al., 2013). Namely, starch is a promising hydrophilic ingredient for scaffolds, which has been reported to be able to regulate the degradation rate of some synthetic polymer-based systems and to control the release of bioactive agents including proteins (Balmayor, Tuzlakoglu, Azevedo, & Reis, 2009; Elvira, Mano, San Román, & Reis, 2002; Gomes, Sikavitsas, Behravesh, Reis, & Mikos, 2003). Starch enzymatically degrades into glucose and non-toxic derivatives and is phagocytized by macrophages (Puppi, Chiellini, Piras, & Chiellini, 2010). Moreover, starch is also a good cell support for osteoblasts since it promotes the adhesion and proliferation of cells and the phenotypic expression of osteoblastic markers (Silva, Coutinho, Ducheyne, Shapiro, & Reis, 2007).

The aim of this work was to implement a $scCO_2$-assisted foaming approach for preparing porous PCL-starch hybrid scaffolds containing PRGF. The processing was carried out in the absence of organic solvents and under mild temperature to avoid problems of cytotoxicity and denaturation of GFs. The direct incorporation of freeze-dried GFs from natural sources in scaffolds by supercritical foaming is herein reported for the first time and applied for GFs of autologous source. Prior to scaffold preparation, starch was pre-processed through thermal gelation as a way to improve PCL:starch matrix homogeneity and final hydrophilicity and ability to control GFs release of the scaffolds. MSCs proliferation in scaffolds containing PRGF was evaluated and compared to the corresponding ones without PRGF. All scaffolds were evaluated regarding their structural and physicochemical properties, PRGF-release properties and cytocompatibility.

2. Materials and methods

2.1. Reagents

PCL (PCLraw, 50 kDa, T_m = 63 °C) in powdered form was from Polysciences (Warrington, PA, USA), native corn starch (St, amylo N-460; 52.6% amylose content, 12.9% loss on drying, conform to USP and EP Pharmacopeias) was from Roquette (Lestrem, France), and carbon dioxide was supplied by Praxair, Inc. (Danbury, CT, USA). Mesenchymal stem cells MesenPRO medium was from Gibco (Life Technologies, Carlsbad, CA, USA); Pierce™ BCA Protein Assay Kit was from Thermo Scientific (Waltham, MA, USA). Human adipose-derived mesenchymal stem cells (ATCC-PCS-500-011) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). TGF-β1 and VEGF ELISA kits were from RayBiotech (Norcross, GA, USA). Fluorescein isothiocyanate (FITC) and trypsin-EDTA solution (0.25%) were from Sigma–Aldrich (St Louis, MO, USA). Lysozyme from chicken egg white was from Fluka (Sigma–Aldrich, St Louis, MO, USA).

2.2. Starch preparation

Starch xerogels were obtained by thermal gelation of a 15% (w/w) starch aqueous solution at two different temperatures (90 °C in an oven or 121 °C in an autoclave) for 20 min. The starch gel was then dried in an oven at 80 °C for 1 day and grinded in a ball mill Mixer Mix MM 400 (Retsch Inc., Newton, PA, USA) to get a white powder (particle size <20 μm). Starch xerogels were denoted as St90 or St121 depending on the gelation temperature used.

2.3. PRGF preparation

PRGF preparation was prepared as previously reported (Diaz-Gomez et al., 2014). Briefly, human PRGF was obtained from human buffy coat (Centro de Transfusión de Galicia, Spain) centrifuged at 400 × *g* for 15 min, and the supernatant rich in platelets (3×10^6 platelets/μL) was collected and poured as 1 mL-aliquots in small tubes. Then, they were activated through four freeze–thaw cycles (−80 °C for 24 h, room temperature for 1 h). Tubes were then centrifuged at 14,000 × *g* for 10 min at 4 °C, and the supernatant containing growth factors was recovered, kept frozen at −80 °C